

# A Single-stranded Nucleic Acid-binding Protein from *Artemia salina*

## I. PURIFICATION AND CHARACTERIZATION\*

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A protein that binds tightly to single-stranded but not to double-stranded nucleic acids has been purified to homogeneity from a high salt wash of ribosomes from cryptobiotic *Artemia salina* gastrulae. The protein, designated HD40 to indicate a helix-destabilizing protein with a molecular weight of 40,000, is present in the high-salt ribosomal wash at a level of about 2 molecules per 80 S ribosome. The protein is monomeric at salt concentrations from 0.01 to 0.5 M and has an  $\alpha$ -helix content of approximately 15%. The amino acid composition of HD40 is characterized by a high glycine content (19.5 mol %), the absence of cysteine, and the presence of the unusual amino acid dimethylarginine. The isolated protein binds preferentially to natural RNA over denatured DNA. HD40 inhibits protein synthesis directed by poly(rU) and by *Artemia* poly(A<sup>+</sup>) RNA in cell-free systems derived from *Artemia* and from wheat germ; inhibition is relieved by excess of mRNA. Single-stranded ribo- and deoxyribopolynucleotides are largely protected from degradation by nucleases when complexed with HD40.

Studies from several laboratories have indicated that RNA-binding proteins analogous to those which bind and destabilize single-stranded DNA helices in the processes of replication and recombination may be involved in the functional expression of various RNA species. Natural single-stranded RNA chains possess a considerable amount of secondary structure represented by double-stranded regions of varying lengths, hairpin loops, bulges, and stacked single-stranded domains. The expression of the biological activity of RNA, e.g. during the initiation and elongation of polypeptides or the transport of messenger ribonucleoprotein particles through the nuclear membrane, may require that part of its secondary structure be disrupted, perhaps transiently, by specific proteins. In prokaryotes, ribosomal protein S1 from *Escherichia coli* and an analogous protein from *Caulobacter crescentus*, which are required for mRNA binding to ribosomes during initiation, were shown to disrupt the secondary structure of various synthetic and natural ribo- and deoxyribopolynucleotides (1-4). A protein from calf thymus, with high affinity for single-stranded DNA and RNA, disrupts the secondary structures of tRNA and 5 S RNA accelerating their conversion into biologically active conformations (5-7). Rabbit reticulocyte initiation factor eIF-3, which is required for the translation of globin mRNA, has been shown to unfold the structure of

globin mRNA and synthetic polynucleotides (8, 9). Conversely, RNA-binding proteins may play a repressory role in translation. A well known example of such a repression is the regulation of translation of individual cistrons of the genome of RNA coliphages by phage-specific coat and replicase proteins (10). Another example is that of the prototype DNA helix-destabilizing protein,<sup>1</sup> the product of phage T4 gene 32, which regulates its own expression by binding to and preventing the translation of gene 32 mRNA (12). In eukaryotes, the role of RNA-binding proteins present in messenger ribonucleoprotein is an unresolved problem. Embryonic systems contain an inactive, protein-rich form of stored mRNA (13, 14). The presence of ribosome-bound inhibitors of protein synthesis in embryonic systems has also been suggested (15, 16).

Embryos of the brine shrimp *Artemia salina* offer a convenient source of components for the study of translation and transcription, as well as providing a useful system for the investigation of control mechanisms during development (16-21). The encysted gastrulae, which are highly stable and metabolically inactive, quickly resume development upon immersion in an aerated salt solution. Although development proceeds in the absence of cell division until the prenauplius stage, an increased level of metabolic activity is denoted by the formation of polysomes in the cytoplasm and increased transcriptional activity in the nucleus. As an approach to the problem of translational control, we have searched for the presence of RNA-binding proteins in the cytoplasm of the cryptobiotic gastrulae of *A. salina*. In this communication, we describe the isolation and partial characterization of a single-stranded nucleic acid-binding protein from undeveloped cysts. A relatively large amount of the protein is found in the ribosomal pellet following high speed centrifugation of the postmitochondrial extract. The protein (HD40,<sup>2</sup> molecular weight 40,000) is a potent inhibitor of protein synthesis *in vitro*. Complexes of HD40 with polynucleotides are largely protected from digestion by several nucleolytic enzymes.

## EXPERIMENTAL PROCEDURES

### Materials

*A. salina* cysts (San Francisco Bay brand) were obtained from Metaframe Corporation, Elmwood Park, N.J. Calf thymus DNA was obtained from Worthington and polyribouridylylate from Miles Laboratories, Inc. Spermine hydrochloride and sodium dodecyl sulfate were obtained from Sigma. Enzyme grade ammonium sulfate, sucrose, and guanidine hydrochloride were purchased from Schwarz/Mann. DEAE-cellulose (DE52) was obtained from Whatman and Sephadex G100 and marker proteins for gel electrophoresis from Pharmacia.

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<sup>1</sup> The term helix-destabilizing protein (HD protein) suggested by Alberts and Sternglanz (11) was designed to avoid confusion with the more recently discovered DNA-unwinding proteins which use the energy of ATP hydrolysis.

<sup>2</sup> The abbreviation used is: HD40, helix-destabilizing protein with a molecular weight of 40,000.

## Buffers

Buffer A contained 20 mM Tris-HCl, pH 7.5, 70 mM KCl (or as indicated in the text), 9 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 M EDTA, and 5% glycerol. Buffer B contained 20 mM Tris-HCl, pH 7.5, 0.5 M KCl (or as indicated in the text), 1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol.

## Standard Unwinding Assay

**Principle**—Poly(rU) forms a helical intramolecular hairpin in the presence of an equimolar amount of spermine (22, 23). The helix-coil transition is highly cooperative, has a  $T_m$  of 29°C, and is accompanied by a 50% hyperchromic effect at 260 nm. RNA helix-destabilizing proteins unfold stoichiometrically and instantaneously the ordered form of poly(rU) at 0°–10°C or prevent the formation of the ordered form upon addition of spermine. The extent of the hypochromic effect upon the addition of spermine to a mixture of poly(rU) and the HD protein (33% at 260 nm in the absence of the HD protein) is a measure of template saturation.

**Procedure**—Measurements were carried out at 10°C in a Zeiss PM6 spectrophotometer, and the chamber was flushed with a stream of dry nitrogen to prevent fogging. Samples of 150 to 300  $\mu$ l were analyzed reproducibly in matched microcuvettes (10-mm pathlength, 1.5-mm wide chambers). Poly(rU) (50  $\mu$ l of a 60.0  $\mu$ M solution in a buffer containing 5 mM Tris-HCl, pH 7.5, and 10 mM NaCl) was mixed with the protein (5 to 60  $\mu$ g), and the volume was adjusted to 300  $\mu$ l with the same buffer. Readings were taken at 260 nm before and after the solution was made 10  $\mu$ M in spermine by the addition of 3  $\mu$ l of 1.0 mM spermine-HCl. The decrease in  $A_{260}$  upon addition of spermine was instantaneous.

**Comments**—The final concentration of NaCl (or KCl) in the assay mixture should not exceed 25 to 30 mM since higher concentrations of salt decrease the  $T_m$  of the poly(rU)-spermine ordered state. Removal of shorter chains from commercial preparations of poly(rU) is sometimes necessary to obtain the full 33% hypochromic effect upon addition of spermine. This is done by reprecipitating poly(rU) at a concentration of 2 to 3 mg/ml from a solution containing 4.0 M NaCl by addition of 0.2 to 0.3 volume of ethanol. Degradation of poly(rU) by nucleases present in the protein sample interferes with the assay. The protein binds stoichiometrically and can be distinguished from nucleolytic activity by increasing the concentration of poly(rU) several-fold while holding the protein concentration constant. Since the assay is rapid (equilibrium is attained within seconds) and can be carried out at 0°C, it is possible to semiquantitatively analyze protein samples containing low levels of nuclease.

## Standard Protein Synthesis Assays

For poly(rU)-directed incorporation, the samples (0.05 ml) contained 24 mM Tris-HCl, pH 7.5, 8 mM magnesium acetate, 80 mM KCl, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 0.16 mg/ml of creatine kinase, 11  $\mu$ M [<sup>14</sup>C]phenylalanine (specific activity, 3.6 mCi/mmol), 40  $\mu$ M each of the 19 amino acids, 0.1  $A_{260}$  unit of yeast tRNA, 0.5  $A_{260}$  unit of *A. salina* high salt washed ribosomes (19), 0.1 mg of *A. salina* high speed supernatant (S105) and poly(rU) and HD40 as indicated in the figure. Incubation was at 25°C for 30 min. For *A. salina* poly(A<sup>+</sup>) RNA-directed incorporation the samples (0.05 ml) contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, 2.5 mM magnesium acetate, 100 mM KCl, 2 mM dithiothreitol, 1 mM ATP, 20  $\mu$ M GTP, 8 mM creatine phosphate, 0.1 mg/ml of creatine kinase, 7  $\mu$ M [<sup>3</sup>H]leucine (specific activity, 2.85 Ci/mmol), 25  $\mu$ M each of the 19 amino acids, 1.35  $A_{260}$  units of wheat germ extract (S30) prepared as described (24); *A. salina* poly(A<sup>+</sup>) mRNA and HD40 were as indicated in the figure. Incubation was at 25°C for 60 min.

## Analytical Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis was performed according to the procedure of Laemmli (25). Protein bands were detected by staining with Coomassie brilliant blue.

*A. salina* Poly(A<sup>+</sup>) mRNA

Total RNA was prepared from polysomes of developed embryos (16 h at 27°C) by the procedure of Mutukrishnan *et al.* (26). Poly(A<sup>+</sup>) mRNA was subsequently obtained by chromatography on oligo(dT)-cellulose (27).

## RESULTS

## Purification of HD40 from Undeveloped Cysts

Crude ribosomes were prepared from 500 g of dry cysts which were pretreated as described (18, 28). Hydrated cysts were ground at 4°C in a mortar with the gradual addition of Buffer A to a final concentration of 2 ml of Buffer A/g of cysts. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 16,000 rpm for 15 min in the Sorvall SS-34 rotor. The supernatant was filtered through glass wool and clarified by centrifugation for 30 min at 50,000 rpm in the Spinco 60 Ti rotor. The crude ribosomal pellet (approximately 7,500  $A_{260}$  units) used as the source of HD40 was obtained by centrifugation of the supernatant for 4 h at 50,000 rpm in the Spinco 60 Ti rotor.

**Step 1**—The ribosomal pellet was suspended in 80 ml of Buffer A containing 0.8 M KCl and stirred for 3 h at 4°C. The ribosomes were then pelleted by centrifugation at 28,000 rpm for 12 h (Spinco 60 Ti rotor), and the clear supernatant fluid was collected.

**Step 2**—The concentration of KCl in the ribosomal wash was adjusted to 0.5 M with Buffer A containing no KCl, and ammonium sulfate was added to 40% saturation. The precipitate which contains the bulk of HD40 was dissolved in Buffer B and dialyzed against the same buffer.

**Step 3**—HD40 was further purified by sucrose gradient sedimentation (Fig. 1). Preparative runs were made using the Spinco 60 Ti rotor by layering 1.5 ml of Step 2 protein (10 mg/ml) on a linear 24-ml 10 to 30% sucrose gradient in Buffer B and centrifuging for 16 h at 32,000 rpm. Helix-destabilizing activity (measured by the standard unwinding assay) was found in the slowest sedimenting peak (*hatched area* of Fig. 1). Active fractions were combined, and the protein was precipitated by 80% saturation with ammonium sulfate; the precipitate was dissolved in 5.0 ml of Buffer B and dialyzed against the same buffer.

**Step 4**—The concentration of KCl in the sucrose gradient fraction was adjusted to 100 mM with Buffer B containing no KCl, and the protein was applied to a denatured DNA-agarose column (1.5  $\times$  13 cm, containing 35 to 40 mg of calf thymus DNA, Ref 29), equilibrated with Buffer B containing 100 mM KCl. The column was washed with the same buffer until most ultraviolet-absorbing material had been eluted, and the bound protein was eluted stepwise with Buffer B containing 250 and 500 mM KCl. HD40 was the predominant protein in the 500

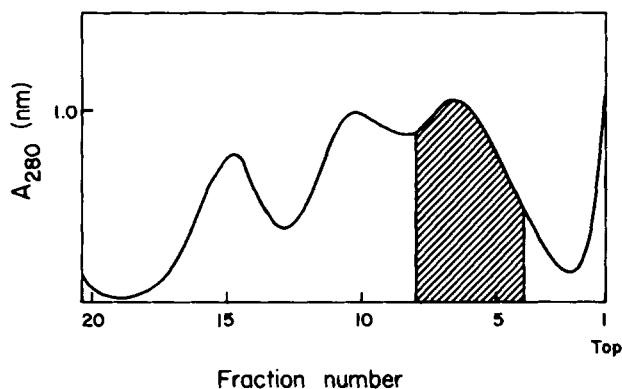


FIG. 1. Sucrose density gradient centrifugation of Step 2 protein. Five milligrams of protein in Buffer B (0.5 ml) were layered on a linear 10-ml 10 to 30% sucrose gradient in the same buffer. Centrifugation was for 21 h at 40,000 rpm at 4°C in a Spinco SW 41 rotor. *Hatched area* shows fractions active in the standard unwinding assay.

mm eluate (Fig. 2); active fractions were combined and concentrated by vacuum dialysis in Buffer B.

**Step 5**—The protein from Step 4 (2.4 ml, 5 mg of protein/ml) was applied to a Sephadex G100 column (1.6 × 95 cm) equilibrated with Buffer B containing 200 mM KCl and the column eluted with the same buffer at a rate of 5.5 ml/h. Two peaks of protein were eluted from the column, the second of which contained HD40 of greater than 95% purity. The purification of HD40 is summarized in Table I. Most of the experiments in this paper were carried out with Step 5 protein

which is essentially homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) and has an  $A_{280}/A_{260}$  ratio of 1.73. Step 5 protein has no detectable exonucleolytic activity toward [ $^3\text{H}$ ]poly(rU), [ $^{14}\text{C}$ ]poly(rA), and [ $^3\text{H}$ ]phage MS2 RNA when assayed according to Spahr (31). No endonucleolytic activity was observed when Step 5 protein was incubated with MS2 RNA and with phage  $\phi\text{X174}$  DNA, and the mixture was analyzed on sucrose gradients (see accompanying paper). No loss of activity was observed upon storage of Step 5 protein in Buffer B (100 mM KCl) at  $-24^\circ\text{C}$  for at least 7 months.

**Step 6**—Some of the data presented in this and the accompanying paper was obtained using an HD40 preparation which was further purified on DEAE-cellulose (HD40 is not retained by DEAE-cellulose at neutral pH at 100 mM KCl). Approximately 1.2 mg of protein were recovered in the flow through when 0.16 ml of Step 5 protein (10 mg/ml) was applied to a 0.2-ml DEAE-cellulose column equilibrated with Buffer B containing 100 mM KCl.

#### Comments on the Purification Procedure

HD40 could not be assayed in the high-salt ribosomal wash and the 0 to 40% ammonium sulfate fraction of the wash because of the presence of nucleases. Sucrose gradient sedimentation (Step 3) was essential since it yielded a protein preparation highly enriched in HD40 which could be assayed using the standard assay procedure. Omitting this step yields a final product of about 80 to 85% purity. Although HD40 has a high affinity for both single-stranded DNA and single-stranded RNA (see below), DNA-agarose rather than RNA-cellulose was used as an affinity matrix in Step 4 since this material could be reutilized many times whereas the RNA was largely degraded after 1 to 2 runs. The total nucleotide content of the single-stranded DNA column employed (100 to 200  $\mu\text{mol}$  of nucleotide) was estimated to exceed by 25- to 40-fold the nucleic acid-binding capacity of the protein applied to the column. This, and the fact that very small amounts of protein are eluted from the column with buffer containing a concentration of KCl greater than 0.5 M provide reasonable assurance that no major single-stranded nucleic acid-binding protein was missed in the course of purification.

The yield of HD40 at Step 3 from a pellet containing 174 nmol of crude *A. salina* ribosomes is about 320 nmol. This value is based on an estimate from the standard assay that HD40 is approximately 15% pure at this stage. It appears that HD40 is a major constituent of cellular protein since its molar ratio with respect to 80 S monosomes is about 2.

The isolation of HD40 from the high-salt extract of crude ribosomes is no more than an indication of its cytoplasmic location since the preparation of the extract may involve considerable damage to the nuclei (see under "Experimental Procedures"). Since the protein exhibits high affinity for both single-stranded DNA and RNA, it was of interest to determine whether it is present in nuclei. *A. salina* nuclei were prepared in sucrose medium as described (21), lysed by freezing and thawing, and the mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only a trace amount of protein was observed to comigrate with authentic HD40. In a similar experiment, HD40 was not detected in a 3.0 M KCl, 5 mM EDTA extract of the membrane fractions obtained from the initial low-speed centrifugation of the homogenate.

#### Physical Characterization of HD40

The molecular weight of HD40, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32), is 40,000 (Fig. 2). A molecular weight of 43,000 was determined

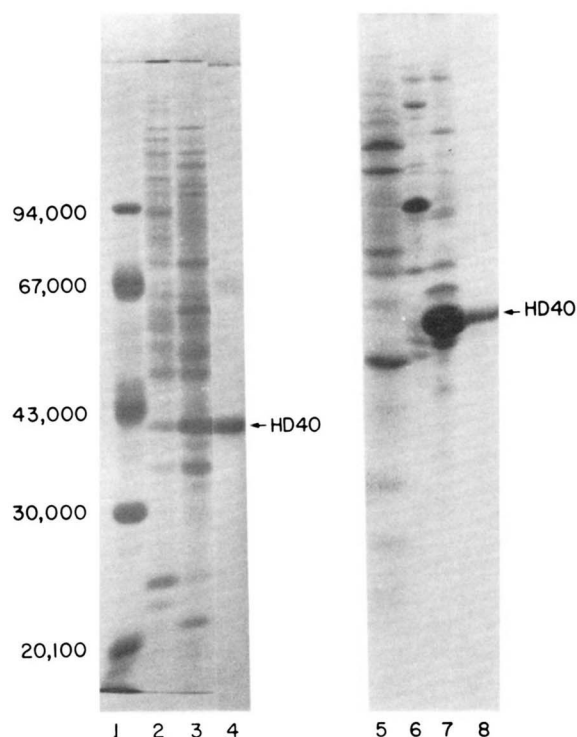


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions in the purification of HD40. Channel 1, marker proteins (from top to bottom), phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor; channel 2, Step 2 protein; channel 3, Step 3 protein; channel 4, Step 5 protein; channel 5, 100 mM KCl eluate from DNA-agarose; channel 6, 250 mM KCl eluate from DNA-agarose; channel 7, 500 mM KCl eluate from DNA-agarose (Step 4 protein); and channel 8, Step 5 protein.

TABLE I  
Purification of HD40 from ribosomal pellet

	Vol- ume	Protein concen- tration <sup>a</sup>	Total pro- tein	Activ- ity <sup>b</sup>	Approx- imate pu- rity
	ml	mg/ml	mg		%
Step 1. High salt ribo- somal wash <sup>c</sup>	70	n.d. <sup>d</sup>	n.d.	n.d.	n.d.
Step 2. Ammonium sulfate precipitation (40%)	15	19	285	n.d.	n.d.
Step 3. Sucrose gra- dient sedimentation	5.4	16	87	40–65	15
Step 4. DNA-agarose chromatography	2.4	5	12	180–240	80
Step 5. Sephadex G100 chromatogra- phy	0.8	10	8	260	95

<sup>a</sup> Determined by the method of Lowry *et al.* (30).

<sup>b</sup> Picomoles of nucleotide (as poly(rU)) prevented from forming helical hairpin per microgram of protein in standard assay.

<sup>c</sup> Obtained from 7500  $A_{260}$  units of ribosomes.

<sup>d</sup> n.d., not determined.

from molecular sieve experiments on a calibrated Sephadex G100 column (0.9 × 58 cm) equilibrated with Buffer B containing 100 mM KCl, indicating that under these conditions HD40 exists as a monomer. Sedimentation equilibrium experiments (Spinco, model E) performed at 10°C in 10 mM Tris-HCl, pH 7.5, containing 50 or 500 mM NaCl or in 7.0 M guanidine hydrochloride gave molecular weight values of 40,000, 42,000, and 44,000, respectively. No polydispersity was observed at HD40 concentrations of up to 1.0 mg/ml. A partial specific volume of 0.72 mg/g estimated from the amino acid composition was assumed for the calculations. A sedimentation coefficient of 2.4 S was determined in buffer containing 20 mM Tris-HCl, pH 7.5, and 100 mM NaCl. The absorptivity of HD40,  $E_{1\text{cm}}^{1\%}$ , is 7.7 at 280 nm.

HD40 has a relatively low content of  $\alpha$ -helix as estimated from circular dichroic spectra obtained with a Cary 61 spectrophotometer and interpreted according to Bevely *et al.* (33). Values of 14 and 17%  $\alpha$ -helix were determined in buffers containing 5 mM Tris-HCl and 10 or 100 mM NaCl, respectively. The CD spectrum was not perturbed by the addition of magnesium ions to the system.

The amino acid composition of HD40 is presented in Table II. Glycine is the most abundant amino acid and together with proline they constitute 24% of the total residues which is in agreement with the low content of  $\alpha$ -helices. The protein is devoid of cysteine and has a low level of tryptophan. An unusual amino acid elutes between ammonia and arginine in the same position as dimethylarginine.<sup>3</sup> A single homogeneous peak is eluted in this position when dimethylarginine is added to the acid hydrolysate of HD40. The high content of glycine, the absence of cysteine, and the presence of dimethylarginine are all typical for a class of low molecular weight (30,000–40,000) RNA-binding proteins isolated from a number of eukaryotic cells (37, 38). The amino acid content of HD40 differs significantly from that of the HD proteins isolated from calf thymus (6). Curiously, the amino acid composition of HD40, except for the presence of dimethylarginine, is quite similar to that of the HD protein from *E. coli* (subunit molecular weight 19,500, Ref. 39).

#### Binding of HD40 to Polynucleotides

Since proteins bind to Millipore filters while polynucleotides do not, the binding of HD40 to polynucleotides can be quantitated by measuring the retention of labeled polynucleotide on the filters in response to the addition of the protein. Table III presents data on the binding of HD40 to polynucleotides in 5 mM Tris-HCl buffer containing 50 mM NaCl and in the same buffer supplemented with  $\text{MgCl}_2$ . In the absence of  $\text{Mg}^{2+}$  ions there is good binding to nearly all the single-stranded ribo- and deoxyribopolynucleotides. Binding is diminished in the presence of  $\text{Mg}^{2+}$  ions with the exception of poly(rU). Elevated ionic strengths also reduce the binding of HD40 to polynucleotides, *e.g.* binding to poly(rU) decreased by 10 and 70% at 0.5 and 0.8 M NaCl, respectively, relative to the level of binding obtained at 50 mM NaCl (data not shown). Similarly, the binding properties of other HD proteins have also been shown to be sensitive to the levels of mono- and divalent cations. The rate of binding is rapid, being essentially complete in seconds. HD40 does not bind to duplex DNA and synthetic duplex RNA. The dependence of the binding of [<sup>3</sup>H]MS2 RNA to Millipore filters on the concentration of HD40 is shown in Fig. 3. A stoichiometry of about 25 nucleotides per protein molecule can be calculated from the data of

<sup>3</sup> Symmetrical  $\text{N}^G, \text{N}^G$ -dimethylarginine and asymmetrical  $\text{N}^G, \text{N}^G$ -dimethylarginine standards are not resolved by the column employed (34).

TABLE II

#### Amino acid composition of HD40

Amino acid analysis was carried out according to Lai *et al.* (34).

Amino acid	Moles of amino acid/mol of protein
Lysine	30.2
Histidine	7.1
Arginine	8.7
Tryptophan <sup>b</sup>	3.7
Aspartic acid, asparagine	41.2
Threonine	15.3
Serine	19.8
Glutamic acid, glutamine	44.5
Proline	17.8
Glycine	74.5
Alanine	28.6
Half-cystine <sup>c</sup>	0
Valine	21.4
Methionine	5.9
Isoleucine	14.0
Leucine	15.0
Tyrosine	21.7
Phenylalanine	16.0
Dimethylarginine	5.3

<sup>a</sup> A monomer molecular weight of 40,000 was assumed for HD40.

<sup>b</sup> Determined spectrophotometrically (35).

<sup>c</sup> Determined as cysteic acid after performic acid oxidation (36).

TABLE III

#### Binding of HD40 protein to labeled natural and synthetic polynucleotides

Labeled polynucleotide (0.6 to 1.0 nmol) was mixed with HD40 in a volume of 0.05 ml at room temperature, incubated for 1 min at 0°C, diluted to 1.0 ml with the corresponding ice-cold buffer, and filtered through Millipore filters. The filters were washed three times with 2.0 ml of buffer. The molar ratio of protein to nucleotide was kept at approximately 1:15. For the binding of poly(rU), poly(rA) and SV40 DNA filters were pretreated with 0.5 KOH (40) to reduce blanks without HD40.

	Polynucleotide retained on filter (% input)	
	5 mM Tris-HCl, pH 7.4, 50 mM NaCl	5 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM $\text{MgCl}_2$
MS2 RNA	100	30
Poly(rU)	75	90
Poly(rA)	56	21
Poly(rC)	70	52
tRNA	26	1
Ribosomal RNA	76	12
SV40 DNA (heat denatured)	85	43
Poly(dA)	61	37
Poly(rA) + poly(rU) (1:1)	5	1
SV40 DNA	3	2

Fig. 3. This value is larger than the stoichiometry determined by optical methods (about 12 to 15 nucleotides, see accompanying paper) and most probably indicates that the HD40-MS2 RNA complex binds to the filter when not fully saturated. The Millipore filter assay was also used to measure the relative affinity of the protein for various polynucleotides. Table IV shows the results of competition experiments in which an excess of unlabeled polynucleotide was mixed with [<sup>3</sup>H]MS2 RNA prior to the addition of HD40. Among the homopolymers assayed, those containing U and T competed most effectively for the protein. Although the nature of the sugar moiety generally had little effect on the binding, poly(rA) showed some affinity for HD40 whereas poly(dA) did not. Significantly, denatured DNA did not compete effectively with [<sup>3</sup>H]MS2 RNA, nor did duplex DNA or synthetic duplex RNA.

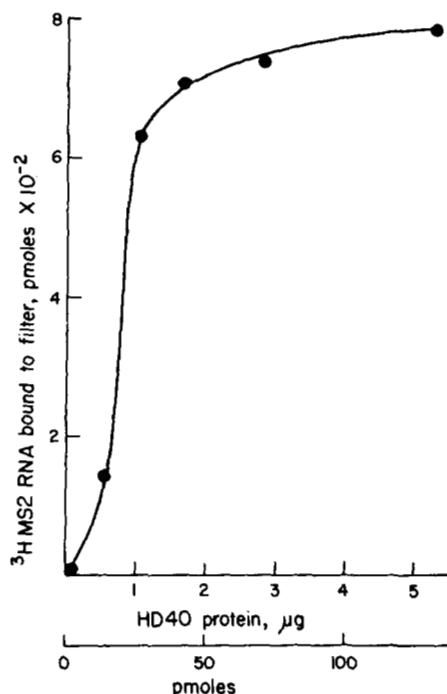


FIG. 3. Binding of HD40 to  $[^3\text{H}]\text{MS2}$  RNA. Labeled polynucleotide (0.8 nmol) was mixed with the protein in 0.1 ml of a buffer containing 5 mM Tris-HCl, pH 7.4, 50 mM NaCl, at room temperature and processed as described in Table III.

TABLE IV

Competition of polynucleotides for HD40 protein

Binding was carried out at 23°C in 5 mM Tris-HCl, pH 7.4, 50 mM NaCl.  $[^3\text{H}]\text{MS2}$  RNA (0.8 nmol) was mixed with unlabeled polynucleotide (2.5 nmol of a single-stranded and 5.0 nmol of double-stranded) in 0.1 ml. HD40 (3 μg) was added, and the samples were filtered through Millipore filters.

Competing polynucleotide	$[^3\text{H}]\text{MS2}$ RNA bound to filter (% input)
None	100
rU	28
rC	100
rA	79
rI	93
dT	31
dU	33
dC	100
dA	100
dI	95
ss SV40 DNA	71
ds SV40 DNA	96
rA + rU (1:1)	100

Inhibition of *in Vitro* Protein Synthesis by HD40

The high affinity of HD40 for single-stranded RNA prompted us to examine its effect on protein synthesis *in vitro*. Experiments were carried out in a complete *A. salina* system with poly(rU) as mRNA and in a wheat germ system directed by *A. salina* poly(A<sup>+</sup>) RNA, the limiting component in both systems being the mRNA. As seen from Fig. 4, addition of HD40 to either system results in a marked inhibition of translation. The inhibition is largely relieved by an excess of mRNA, e.g. at an HD40:poly(A<sup>+</sup>) RNA nucleotide ratio of 1:65 incorporation is inhibited by about 60% whereas at a ratio of 1:160, the inhibition decreases to approximately 7% (Fig. 4B). Actual binding of HD40 to poly(A<sup>+</sup>) RNA may be even lower than that calculated from input concentrations due to the presence of  $\text{Mg}^{2+}$  ions in the incorporating system (cf.

Table III). In other experiments (not shown) it was found that an excess of either the S100 fraction or the ribosomes in the presence of a limiting amount of mRNA does not detectably reduce the inhibition of incorporation by protein HD40. These observations suggest that the protein binds to mRNA in a manner which prevents the normal translational process.

Influence of HD40 on Nucleolytic Digestion

The nucleolytic activity of pancreatic ribonuclease toward poly(rU) and MS2 RNA is significantly reduced in the presence of protein HD40 (Fig. 5). The protective effect is seen at protein concentrations less than those required to saturate the template but greater than about one protein molecule per 30 nucleotides. The diminution of nucleolytic activity is inversely related to the concentration of HD40 and reaches a maximum value at a protein:nucleotide ratio of 1:7. Complex formation of several RNA and DNA substrates with HD40 leads to marked protection from digestion by snake venom phosphodiesterase and pancreatic deoxyribonuclease (Table V). However, complexing with HD40 provides little or no template protection from digestion by micrococcal nuclease (Table V).

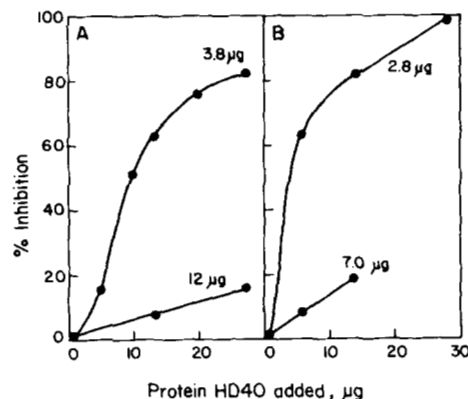


FIG. 4. Inhibition of *in vitro* protein synthesis by HD40. A, poly(rU) directed; B, *A. salina* poly(A<sup>+</sup>) RNA directed. Amounts of mRNA added are indicated on the graphs.

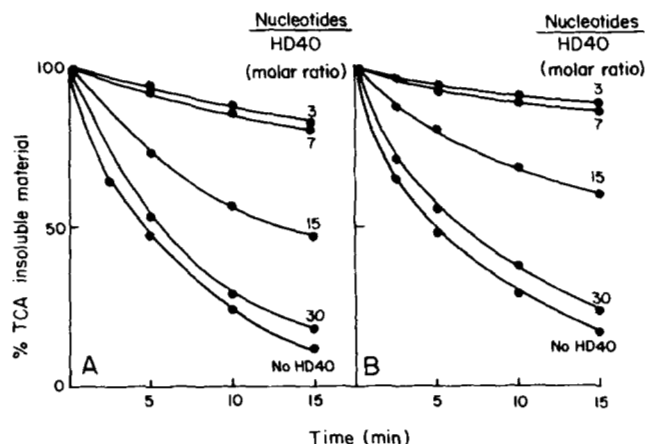


FIG. 5. Degradation of  $[^3\text{H}]\text{MS2}$  RNA and  $[^3\text{H}]\text{poly(rU)}$  by pancreatic ribonuclease in the presence of HD40. A,  $[^3\text{H}]\text{MS2}$  RNA (0.5 nmol of nucleotide) in 0.1 ml of buffer of Table V was mixed with the protein, and RNase (2 ng/ml) was added. B,  $[^3\text{H}]\text{poly(rU)}$  (0.73 nmol) in 0.1 ml of the same buffer was mixed with the protein, and RNase (1 ng/ml) was added. Incubation was at 30°C. See Table V for the composition of the buffer and other details. TCA, trichloroacetic acid.

TABLE V

## Template protection by protein HD40

The amount of enzyme used was that which gave 85 to 90% acid-soluble radioactivity in 15 min at 30°C in the absence of HD40. Labeled polynucleotide (0.5 to 1.0 nmol) was mixed with HD40 protein in a buffer containing 5 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.01 mM MgCl<sub>2</sub> in a volume of 0.1 ml prior to the addition of the enzyme. The molar ratio of nucleotide:protein was kept at 8:1. The reaction was terminated by adding 0.2 ml of 0.5 M HClO<sub>4</sub> and 100 µg of yeast RNA (reaction mixtures containing poly(rU) were precipitated with 10% trichloroacetic acid). The mixtures were held in ice for 10 min, centrifuged at low speed (5 min at 8000 rpm), and an aliquot of the supernatant was neutralized with NaOH and counted in Bray's solution.

	Percentage of protection <sup>a</sup>		
	Snake venom phosphodiesterase	Pancreatic DNase I	Micrococcal nuclease
	%		
MS2 RNA	91		0
Poly(rU)	70		
Poly(rA)	84		32
SV40 DNA denatured	85.5	78	26
Poly(dA)	62		0

<sup>a</sup> The percentage of decrease in acid-soluble radioactivity in the presence of HD40 after 15 min of incubation at 30°C was taken as the measure of protection.

## DISCUSSION

The mechanisms by which translational processes are regulated in unfertilized eggs as well as in undeveloped embryos are not understood. A masked form of mRNA, translatable *in vitro* after the removal of its associated proteins, is known to be present in undeveloped cysts of *A. salina* (19, 41). Likewise, the ribosomes from undeveloped cysts are largely nonfunctional, but in this case *in vitro* activity depends to a considerable extent on the isolation procedure and can be greatly increased by washing the ribosomes with buffers containing high concentrations of salt (16). Our search for RNA-binding proteins potentially involved in the regulation of translation resulted in the isolation of a protein having a monomeric molecular weight of 40,000 from the crude ribosomal pellet obtained from undeveloped cysts. The protein exhibits no tendency for self aggregation although aggregates are formed in the presence of polynucleotide templates (see accompanying paper). HD40 has a relatively low content of  $\alpha$ -helices, it is rich in glycine, devoid of cysteine, and it contains the unusual amino acid dimethylarginine. The amino acid composition of HD40 resembles closely that of a group of RNA-associated nuclear proteins from rat liver (37) and HeLa cells (38). HD40 binds strongly to both single-stranded RNA and DNA but not to native DNA or synthetic duplex RNA. In the accompanying paper, we show that the interaction of HD40 with both natural and synthetic polynucleotides brings about considerable disruption of their ordered structures. In this respect, protein HD40 is analogous to ribosomal protein S1 from *E. coli* and to other known nucleic acid helix-destabilizing proteins (1, 2). Inhibition of *in vitro* protein synthesis by HD40 at limiting levels of mRNA is also similar to the behavior of protein S1 (42).

The binding of HD40 to polynucleotide templates appears to be largely unspecific (Table III), although competition experiments (Table IV) indicate that natural RNA is preferred over single-stranded DNA. The higher affinity for RNA and its cytoplasmic location argues against the possibility of HD40 being a DNA helix-destabilizing protein involved in replication. The competition experiments of Table IV seem to indicate a peculiar preference of HD40 for U- and T-containing

polynucleotides, regardless of the sugar moiety. However, it should be pointed out that all those polymers which compete effectively with [<sup>3</sup>H]MS2 RNA for HD40, *vis.* poly(rU), poly(dU), and poly(dT), are nearly completely unstacked random coils under the conditions of the assay, and their high affinity for the protein may be related to their conformation rather than to any base preference in binding.

The relative abundance of HD40 in the cytoplasm, its inhibitory behavior on *in vitro* protein synthesis, and its protective effect against nucleolytic digestion suggest that the protein may be involved in the regulation of translation. Further work, *e.g.* on the *in vivo* association of HD40 with ribosomes or with messenger ribonucleoprotein particles, will be required to establish the extent of its involvement as a regulatory element in translation.

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